

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

**UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office**

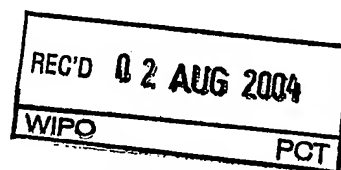
July 28, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: 60/465,149

FILING DATE: April 24, 2003

RELATED PCT APPLICATION NUMBER: PCT/US04/12354



**By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS**



T. Wallace
T. WALLACE
Certifying Officer

**PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)**

BEST AVAILABLE COPY

84-25-03 60455149-A/

Please type a plus sign (+) inside this box 

PTO/SB/16 (02-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

PROVISIONAL APPLICATION FOR PATENT COVER SHEET This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EU 861166840US

INVENTOR(S)					
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)			
Lisette William C.	Acevedo Sessa	New Haven, CT Madison, CT			
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
Nogo is a novel regulator of endothelial cell function and vessel remodeling					
Direct all correspondence to:		CORRESPONDENCE ADDRESS			
<input type="checkbox"/> Customer Number 		<div style="border: 1px solid black; padding: 5px; display: inline-block;"> Place Customer Number Bar Code Label here </div>			
OR		Type Customer Number here			
<input checked="" type="checkbox"/> Firm or Individual Name	Yale University, Office of Cooperative Research				
Address	433 Temple Street				
Address					
City	New Haven	State	CT	ZIP	06511
Country	USA	Telephone	(203) 436-8096	Fax	(203) 436-8086
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages		29	<input type="checkbox"/> CD(s), Number		
<input type="checkbox"/> Drawing(s) Number of Sheets			<input type="checkbox"/> Other (specify)		
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE AMOUNT (\$) <div style="border: 1px solid black; padding: 10px; width: 100px; margin: 0 auto;">\$80.00</div>	
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees					
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:		25-0110			
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input type="checkbox"/> No.					
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are:					
NIH HL57665, HL61371 and HL64793					

Respectfully submitted,

SIGNATURE 

TYPED or PRINTED NAME L. Alan Carr, Jr.

TELEPHONE (203) 785-3074

Date 04/23/03

REGISTRATION NO.
(if appropriate)
Docket Number:

NA

1492

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

Nogo is a novel regulator of endothelial cell function and vessel remodeling

Lisette Acevedo¹, Jun Yu¹, Hediye Erdjument-Bromage², Robert Qing Miao¹, Ji-Eun Kim³, David Fulton¹, Paul Tempst², Stephen M. Strittmatter³ and William C. Sessa¹

¹Department of Pharmacology and Program in Vascular Cell Signaling and Therapeutics, Boyer Center for Molecular Medicine, ³Neurology and Neurobiology, Yale University School of Medicine, New Haven, CT and ²Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA

Address for correspondence:

William C. Sessa, Ph.D.

Yale University School of Medicine

Boyer Center for Molecular Medicine

295 Congress Avenue

New Haven CT 06536-0812

Phone :203-737-2291; Fax :203-737-2290; email:william.sessa@yale.edu

The peripheral roles of Nogo are virtually unknown. Here, we identify Nogo-B as a component of lipid raft domains in cultured endothelial cells. In contrast to the inhibitory action of Nogo-A on cell adhesion and axonal sprouting, the amino terminus of Nogo-B promotes the adhesion, spreading and migration of endothelial cells. We also show that Nogo-B is highly expressed in intact blood vessels plays a role in vascular remodeling. Vascular injury in Nogo-A/B knockout mice promotes exaggerated neointimal proliferation and conversely adenoviral mediated transduction of injured blood vessels with Nogo-B reduces neointimal expansion in wild-type mice. Thus, Nogo-B is a novel regulator of vascular homeostasis and remodeling, broadening the functional scope of this family of proteins.

The Nogo isoforms -A, -B and -C are members of the reticulon family of proteins. Nogo-A and Nogo-C are highly expressed in the CNS, with Nogo-C additionally found in skeletal muscle, while Nogo-B is found in most tissues.[1, 2] Nogo-A, produced in oligodendrocytes, is an inhibitor of axonal growth and repair. Neutralization of Nogo-A function with antibodies that target the amino terminus [3, 4] or receptor blockade with a peptide that antagonizes the loop domain of Nogo-A (Nogo-66) from binding to its cognate receptor NgA-R[5], promotes axon regeneration after injury in mice. Given the considerable implications of understanding Nogo-A mediated processes in the nervous system, little attention has been paid to the function or significance of Nogo isoforms in non-neural tissues.

Since our lab is interested in signaling processes arising in caveolin-1 enriched microdomains (CEM) and/ or lipid rafts (LR), we performed a proteomic analysis of highly purified caveolin-1 containing, cholesterol-rich, buoyant membrane microdomains (CEM/LR) isolated from endothelial cells and identified Nogo-B in these membrane subfractions. As seen in Fig. 1A, RT-PCR of total RNA isolated from a human endothelial cell line (EA.hy.926) and primary cultures of human umbilical vein endothelial cells (HUVEC) shows the expression of Nogo-A, total Nogo-B and Nogo-B2 mRNAs in endothelial cells (plasmids encoding the cDNAs for human Nogo-A and -B are positive controls). Western blotting with an antibody that recognizes amino acids 1-18 in the amino terminus of both Nogo-A and -B (α -Nogo Ab 1-18) documents endogenously expressed Nogo-B protein in COS cells transfected with plasmids encoding Nogo-A or Nogo-B and in endothelial cells, with Nogo-A protein found only in cells transfected with the Nogo-A cDNA (Fig 1B). Similar results have been obtained documenting mRNA, but not protein expression of Nogo-A in several tissues by other investigators.[6] Sucrose gradient fractionation of endothelial cell membranes into buoyant, caveolin-1 enriched microdomains (fractions 2-4, enriched in caveolin-1) versus other membranes enriched in angiotensin converting enzyme (ACE), a bulk plasma membrane marker and β -COP, a Golgi/post-Golgi vesicle marker, demonstrates enrichment of Nogo-B in light membrane fractions (1C). To assess if Nogo-B is in lipid rafts of the plasma membrane (PM) versus other organelles, we first isolated PM, then isolated CEM/LR by sucrose gradient fractionation. As seen in Fig 1D, Nogo-B, caveolin-1, ACE and calnexin (an endoplasmic reticulum marker protein) were found in the starting material (post-nuclear supernatant, PNS). After isolation of PM, Nogo-B,

caveolin-1 and ACE are present in the PM pellet and de-enriched in the post membrane supernatant (PMS). Residual Nogo-B is found PMS, co-sedimenting with calnexin, consistent with the previously described localization of Nogo-A protein in the endoplasmic reticulum. Further isolation of buoyant density membranes from the PM fraction (LR), established by the presence of caveolin-1 and absence of ACE and calnexin, identifies Nogo-B in plasmalemma CEM/LR. This is further supported by immunofluorescent microscopy demonstrating partial co-registration of Nogo-B and caveolin-1 in permeabilized endothelial cells (Fig 1E) showing a predominantly reticular pattern in permeabilized cells with some plasmalemmal staining.

To determine the orientation of Nogo-B in the PM, we performed FACS analysis in non-permeabilized EA.hy.926 cells with α -Nogo (1-18) and non-immune goat primary IgG as a control for the Nogo-B antibody. Fig.1F demonstrates the presence of Nogo-B on the cell surface. This reflects approximately 1-2 % of the total Nogo-B pool, relative to the population of Nogo-B in permeabilized cells. To examine this pool microscopically, immunofluorescent microscopy for Nogo-B was performed in non-permeabilized cells. As seen in Fig 1G (upper left panel), α -Nogo (1-18) labels the cell surface of endothelial cells in a punctate manner consistent with patching of protein on the cell surface, whereas labeling with an antibody against caveolin-1 (an intracellular membranous antigen) is absent (upper right panel). The cell surface labeling of Nogo-B is eliminated by the immunogenic peptide (bottom left panel, 1G) and with goat IgG (bottom right panel). Collectively, these data identify Nogo-B as a protein found in plasmalemma CEM/LR of endothelial cells with its amino terminus oriented toward the cell surface.

Since the amino terminus of Nogo-B appears oriented extracellularly in endothelial cells, we examined the potential functional domains of Nogo-B using glutathione S-transferase fusion proteins encompassing the amino terminal domain (residues 1-200, GST Am-Nogo-B) and the loop domain (residues 236-301, GST Nogo-66; Fig. 2A). These regions are conserved in Nogo-A and -B, with the amino terminus of Nogo-B being much shorter. A fusion protein of the longer amino terminus of Nogo-A (residues 1-1040) inhibits the spreading of various cells, whereas the Nogo-66 specifically inhibits the spreading of neurons.[7] To characterize the activity of Nogo-B on endothelial cell function, we examined if GST Am-Nogo-B or GST Nogo-66 influenced endothelial cell spreading. While coating with GST Am-Nogo-B reduces spreading of COS cells as seen previously, it increases endothelial cell spreading. GST Nogo-66 has no significant effect on spreading of either cell type (Figs 2B and C). Next, we examined the effects of Am Nogo-B on endothelial cell adhesion. Coating plates with GST-Am Nogo-B, but not GST alone, also increases the adhesion of both EA.hy.926 cells and HUVEC. Finally, since Am Nogo-B increases the spreading and adhesion of endothelial cells, necessary processes for cellular migration, we examined if soluble Am Nogo-B may act as a chemoattractant for endothelial cells. HUVEC were cultured onto transwell inserts and GST, GST-AmNogo-B or vascular endothelial growth factor (VEGF, as a positive control) were added to the bottom wells of the chambers to establish a gradient. As seen in Fig 2F, GST Am-Nogo-B, but not GST alone, dose-dependently increases endothelial cell migration. More importantly, adding Am-Nogo-B (Ng/Ng) or VEGF (V/V) to both the upper and lower chambers to eliminate the chemoattractant gradient, abolishes

directional migration. These data show that the amino terminus of Nogo-B (1-200) is a new functional domain for Nogo mediated signaling that promotes endothelial cell spreading, adhesion and migration, processes important for angiogenesis and vascular remodeling.

We next examined the expression of Nogo isoforms in several blood vessels obtained from mice. As seen in Fig 3A, the mRNA for all isoforms (using specific primers for total Nogo-B (Nogo B1 and B2), Nogo-B2, Nogo-A and Nogo-C) were expressed in mouse brain, femoral and carotid arteries and thoracic and abdominal aorta. Western blotting of cells extracts from these tissues documented the expression of Nogo-B protein in isolated blood vessels, but not Nogo-A, which is enriched in brain extracts (Fig. 3B). To examine the tissue distribution of Nogo A/B *in vivo*, we used mice with targeted gene trap of the *nogo ab* locus[8]. The gene trap locus is at the 5' end of the largest Nogo A selective exon disrupting the expression of both Nogo -A and -B. These mice are viable, fertile and exhibit normal behaviors as described and can be used to track the endogenous expression of Nogo A/B due to the insertion of the β -galactosidase gene at the *nogo* locus. As seen in Fig 3C, Nogo A, but not Nogo-B, is highly expressed in brain of wild-type mice (lane 1) whereas Nogo-B, but not Nogo-A, is highly expressed in aorta (lane 2). Disruption of *nogo ab* (-/-) results in the loss of immunoreactive Nogo- B from the aorta (lane 3). Next, we examined the endogenous expression of the *nogo ab* locus in various blood vessels taking advantage of the β -galactosidase gene trap in Nogo A/B (+/-) mice. The *nogo ab* gene is expressed the endothelial and smooth muscle cells of the femoral artery, endothelium of the paired femoral vein (Fig 3D) and both layers of the

aorta (Fig. 3E). In whole mount staining, *nogo ab* is expressed in large blood vessels emanating from the heart, the atria, and main coronary vessels (see arrows in Fig 3F). Cross section through the atria shows the transgene in atrial myocytes, coronary vessels, and more sparsely in ventricular myocytes peripheral to the vessel (Figs. 3 G and H). Additional staining was seen in most vascular tissues including vessels in the brain Fig. 3I), lung and in epithelial cells in the lung, intestine and gall bladder (not shown).

Since Nogo-A/B (-/-) mice are viable, Nogo isoforms are not essential for vascular development; however, Nogo-B expression in the vessel wall suggests that it may influence several aspects of post-natal vascular homeostasis, including response to injury and luminal remodeling. To investigate the role of Nogo-B during vessel injury, femoral arterial wire injury was performed in C57BL/6J mice and immunohistochemistry for Nogo-B examined in control and injured vessels. As seen in Fig. 4A, Nogo-B protein is amply expressed throughout the vessel wall in control vessels and is markedly reduced in vessels at one week post-injury. This suggests that the loss of Nogo-B may influence aspects of vascular remodeling post-injury. To further examine the endogenous role of Nogo-B in vessel remodeling, femoral artery injury was performed in wild-type mice (C57BL/6J), Nogo A/B (+/-) or Nogo A/B (-/-). As seen in Fig 4B, Nogo A/B (-/-) mice exhibit enhanced neointima formation at two weeks post-injury compared to wild-type mice. Quantitative morphometry of injured vessels reveals a "gene-dosage effect" with a progressive increase in neointimal expansion (i.e. increased intimal area and I/M ratio) dependent on the loss of one and two copies of the gene, respectively (Fig 4C). Since wire injury acutely triggers the loss of endothelium, activation of

coagulation/inflammation, smooth muscle apoptosis followed by migration, proliferation and reorganization of endothelium smooth muscle cells and fibroblasts, we examined the the incorporation of BrdU to assess proliferation during the remodeling response two weeks post-injury. The increased cellularity found in injured vessels from the Nogo A/B (-/-) mice is associated with a marked increase in cellular proliferation in primarily the intimal and adventitial layers of the vessel wall (Figs. 4D and E). These data demonstrate that the genetic loss of *nogo ab* exaggerates vascular injury and neointimal proliferation thus providing compelling evidence for a role of endogenous Nogo-B in vascular wall physiology and pathophysiology.

To further test if Nogo-B can regulate injury evoked neointimal expansion, we generated an adenoviral construct expressing an HA-tagged form of Nogo-B (Ad Nogo-B). As seen in Fig. 5A, infection of endothelial cells with increasing titers of Ad Nogo-B results in increased expression of the transgene. Next, we introduced adenoviruses expressing either β -galactosidase (Ad β -gal) or Ad Nogo-B, applied them to the adventitial side of the vessel wall immediately after wire injury using pluronic gels and examined neointima formation. Injured femoral arteries from mice transduced with Ad β -gal exhibited robust neointima formation (arrows in Fig 5B) as quantified by an increase in intimal area and intimal:media ratios (I/M, Fig 5C, open bars) three weeks post injury. In contrast, arteries transduced with Ad Nogo-B revealed a marked reduction in gross injury (right panel, Fig 5B), neointimal area and I/M ratios (Fig 5C, black bars). To determine the Ad Nogo-B effect on the proliferative phase of neointima formation, we examined the incorporation of BrdU into cells one week post-injury. As seen in Fig 4E, transduction of

vessels with Ad Nogo-B (black bars) markedly reduces BrdU incorporation, quantified as BrdU positive cells/total cells per section, in the intima, adventitia of the vessel wall compared to Ad β -gal transduced vessels (open bars). These data suggest that transduction of vessels with Ad Nogo-B rescues the acute loss of Nogo-B, thus preventing neointima formation and abnormal remodeling.

Our study identifies Nogo-B in CEM/LR domains of the plasma membrane of endothelial cells. Although the mRNA for Nogo-A is also present in endothelial cells and blood vessels, only Nogo-B protein can be detected in these extracts. Furthermore, Nogo-B found on the cell surface is oriented with the amino terminus being extracellular. The amino terminus of Nogo-B can induce the *in vitro* spreading, adhesion and migration of endothelial cells in contradistinction to the inhibitory actions of Am Nogo-A and Nogo-66 on cellular motility suggesting that there are multiple functional domains that can promote or inhibit cellular function residing within the N-terminus of Nogo isoforms. Moreover, Nogo isoforms may exert cell and tissue specific effects considering the restricted expression patterns of Nogo -A/B. The biological significance of Nogo-B in blood vessels is underscored by its endogenous expression in arteries and veins found in multiple organs, marked neointimal expansion in injured vessels of Nogo-A/B (-/-) mice and Ad Nogo-B mediated correction of neointimal proliferation and remodeling in injured vessels of wild-type mice. Thus, identification of this novel function of Nogo-B in cells of the vessel wall provides the first *in vivo* evidence for a peripheral role of Nogo isoforms. In addition, the presence of Nogo-B in vessels may enrich our understanding

of the pathways that regulate angiogenesis and tissue remodeling in disease states such as atherosclerosis, post-angioplasty restenosis, diabetes and cancer.

Methods

Cell culture – EA.hy.926 cells were cultured in high glucose DMEM with 10% FBS (Gibco) and HAT (Sigma, St. Louis, MO). HUVECs were cultured in M199 with 20%FBS and ECGS. HEK293T and COS-7 cells were cultured in high glucose DMEM with 10%FBS.

Expression vectors – The full length coding sequence of Nogo-B was amplified from EA.hy.926 through RT-PCR and ligated into pcdna3. GST-Am-Nogo-B was generating by ligating cDNA encoding residues 1-200 of Nogo-B into pGEX4T-1. Ad Nogo-B was generated as previously described by ligating HA tagged full length Nogo-B into pShuttleCMV. [9] GST-Nogo-66, pcdna3.1-MycHis Nogo-A[1] and Ad β -Gal has been described. (Walsh)

RT-PCR –Total RNA from tissue and cells was isolated using RNEasy Kit (Qiagen). RT-PCR was then performed using 100ng and 500ng RNA respectively and primers specific for Nogo A, Nogo B, Nogo B2, Nogo C, and β -actin (Invitrogen, Burlingame, CA). 10ng Nogo-B and Nogo-A plasmid were used as controls.

Western Blot Analysis – COS-7 were cells transfected with pcdna3NogoB and pcdna3.1NgA-MycHis using Lipofectamine 2000 (Invitrogen, Burlingame, CA) as controls. 20ug protein from HUVEC and EA.hy.926 was used. Expression of Nogo was detected using anti-Nogo 1-18 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-

NogoA [10]. Anti- β -actin (Sigma, St. Louis, MO) or anti-hsp90 (BD Biosciences Pharmingen, San Diego, CA) was used to control for loading.

Isolation of CEM/LR and identification of Nogo-B. CEM/LR from proliferating EA.hy.926s were prepared using a sodium carbonate, sucrose density gradient fractionation as described previously[11] with slight modifications. To further purify lipid-rich microdomains, the buoyant, light-scattering membrane fraction from the first gradient was reloaded on a discontinuous sucrose gradient, spun and recollected. CEM/LR were pelleted at 100,000xg, resuspended in 2X Laemli Buffer. Concentrated membranes were run on SDS-PAGE and stained with Coomassie. Visible bands were excised from the gel and prepared for MALDI-ReTOF MS as described previously. Top 'major' experimental masses (m/z) combined from MALDI-ReTOF MS experiments were used to search a non-redundant protein database using the Peptide Search Algorithm. [12, 13] Plasma membrane (PM) CEM/LR were isolated by homogenizing cells in Tris pH 9.6, 5mM $MgCl_2$, and pelleted at 10,000 xg to enrich PM. PM was resuspended in 500mM sodium carbonate and processed as above to isolate CEM/LR.

Surface staining/FACS Analysis – EA.hy.926 cells were incubated with either 5 μ g anti-Nogo antibody or goat IgG for 1hr at 4°C prior to fixation with 3% paraformaldehyde. Cells were then incubated with FITC-anti-goat (JacksonImmuno). For surface staining anti-caveolin-1 antibody (Santa Cruz) as well as peptide used to raise anti-Nogo antibody (Santa Cruz) were used as controls. For caveolin, cells were then incubated with anti-rabbit secondary antibody (JacksonImmuno). Permeabilized cells were treated with 0.1%

Triton-X 100 for 5 min prior to incubation with primary antibody. For FACs analysis cells were detached using Versene (Bio-Whittaker, Walkersville, MD) and equal cell number were treated as above. For permeabilization, 0.3% saponin was added with antibody.

Cell spreading, adhesion and migration assays – 125pmol GST, GST-Am-NogoB, or GST-Nogo66 was added to 0.02% Poly-L-lysine coated 12mm coverslips and let dry overnight. EA.hy.926 cells (50,000) were plated and let adhere onto for 2hrs prior to fixation and staining with Alexa 594-Phalloidin (Molecular Probes). Between 150 and 200 cells were counted per coverslip. For adhesion experiments were performed essentially as described[14], with minor modifications. Titerrek plates were coated as above except 25pmol of protein and 20,000 cells were plated onto each well, let adhere for 1hr prior to fixation and quantitation. For migration experiments, a modified Boyden chamber was used (Costar transwell inserts, Corning Inc, Acton, MA) [15]. The transwell inserts were coated with a solution of 0.1% gelatin (Sigma, St. Louis, MO) in PBS at 4 °C overnight and then air-dried. VEGF at 50 ng/ml (1.1nM) or recombinant GST Am-Nogo-B at different concentrations (100nM, 10nM and 1nM, respectively) dissolved in medium 199 containing 0.1% bovine serum albumin (BSA) was added in the bottom chamber of Boyden apparatus. HUVEC (2×10^5 cells) suspended in 1 μ l aliquot of medium 199 containing 0.1% BSA was added to the upper chamber. After 5 hours incubation, cells on both sides of the membrane were fixed and stained with Diff-Quik staining kit (Baxter Healthcare Corp, Dade Division, Miami, FL). The average number

of cells from five randomly chosen high power (400x) fields on the lower side of the membrane was counted.

X-gal staining – At death, Nogo A/B (+/-) mice were perfusion fixed with 2% paraformaldehyde. Tissues were excised and stained overnight at 37°C. Vessels were embedded in OCT and cryostat frozen sections were obtained for the femoral and aorta (5µm and 10µm respectively) Whole mount pictures of other tissue were taken on a camera mounted on a dissecting scope prior to embedding in paraffin for 5µm sections counterstained with eosin.

Femoral artery injury and adenoviral transduction - All experiments were approved by the institutional animal care and use committees of Yale University. 8-10-wk-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) or Nogo-A/B (+/-, -/-) mice were anesthetized with ketamine/xylazine (79.5 mg/kg ketamine, 9.1 mg/kg xylazine). Femoral artery injury, adenoviral infection and morphometry were performed as previously described. [16, 17] Ad β -Gal or Ad Nogo-B (3×10^8 PFU) was delivered by painting the adventitia side of the femoral artery with 50 μ l of adenovirus (15 μ l)/30% Pluronic-127 gel (35 μ l, Sigma, St. Louis, MO) mixture immediately after injury. Injured femoral arteries were collected 7 days, 2wk and 3 wk after surgery. 5-Bromo-2'deoxyuridine (BrdU) was injected subcutaneously (25 mg/kg) 3 days before sacrifice daily and intraperitoneally (30 mg/kg) 12 h before death. Ad Nogo-B was generated as described.

Histology and immunohistochemistry- At death, perfusion fixed (4% paraformaldehyde in PBS, PH 7.4) or fresh carotid arteries were taken and embedded in O.C.T (Tissue-Tek Elkhart, IN). Cryosections (5 μ m) of arteries were obtained for hematoxylin/eosin, elastic staining and immunohistochemistry (IH). For IH, artery sections were quenched for endogenous peroxidase, blocked in 10% goat or donkey serum and incubated with α -Nogo-B (Santa Cruz) or α -BrdU (Pharmingen). Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed Peroxidase Substrate Kit, Vector Laboratories) and 3-amino-9-ethyl carbazole as chromophore (AEC, Vector). BrdU index was the percentage of BrdU positive cells of total nuclei in intima, media and adventitia.

60165149 0424

Figure 1. Identification of Nogo-B in endothelial cells. A. mRNA for various Nogo isoforms in endothelial cells was detected by RT-PCR using primers for the different isoforms. As controls, COS cells were transfected with plasmids encoding human Nogo-A and -B cDNAs. B. Expression of Nogo proteins in COS cells expressing Nogo-B, Nogo-A and in endothelial cells. COS cells and endothelial cells endogenously express Nogo-B protein, but not Nogo-A protein. Protein expression was monitored using anti-Nogo-A/B antibody and anti-hsp90 for a loading control. C. Enrichment of Nogo-B in CEM/LR in EA.hy.926 cells. Equal volumes (25 μ l) of sample were loaded from each fraction and proteins blotted with anti-Nogo-B, and anti-caveolin 1, angiotension converting enzyme (ACE; QED Biosciences, San Diego, CA) and β -COP (ABR, Golden, CO) to determine the location of these protein markers. D. Nogo-B is present in plasmalemmal CEM/LR of EA.hy.926 cells. Equal amounts of protein (5 μ g) in PNS (post nuclear supernatant), PM (plasma membrane), PMS (post membrane supernatant) were loaded and 50 μ l volume was loaded from isolated LR fraction (1 ml). E. Co-localization of Nogo-B and caveolin-1 in EA.hy.926 detected by immunofluorescence microscopy. Surface labeling of Nogo-B determined by FACS analysis (F) and surface staining of Nogo-B using immunofluorescent microscopy (G).

Figure 2. The amino terminus of Nogo-B promotes endothelial cell functions. A. Diagram of primary amino acid domains of Nogo-B used for generation of GST fusion proteins. B. GST Am-Nogo-B promotes endothelial cell, but not COS-7 cell spreading. Data represent mean \pm SEM from n= 8 (PBS, GST Nogo-66) n=12 (GST, GST Am-Nogo-B). C. Representative Alexa 594-Phalloidin staining of EA.hy.926 cells plated as

in B. **D.** GST Am-Nogo-B promotes adhesion of endothelial cells. Data represent mean \pm SEM (n=4), $p < 0.05$ and this experiment was repeated 3 times with similar results. **E.** GST-Am-Nogo is a chemoattractant for HUVEC. Cell migration was determined in modified Boyden chambers using recombinant GST, GST Am-Nogo-B and VEGF (1.1 nM) respectively. Each value represents mean \pm SEM (n=3), $p < 0.05$ and this experiment was repeated 3 times with similar results.

Figure 3. Nogo-B is present in intact blood vessels. **A.** The mRNAs for various Nogo isoforms were detected in isolated murine blood vessels by RT-PCR using isoform selective primers. **B.** Nogo-B, but not -A, protein expression in murine blood vessels (right panels). Lysates of murine brain or COS-7 cells transfected with Nogo-A or B cDNAs were used as Ab controls and β -actin as a loading control. **C.** Loss of Nogo-A/B proteins in brain and aortic extracts prepared from Nogo-A/B (-/-) mice. X-Gal staining of a femoral artery/vein pair (**D**) and aorta (**E**) isolated from a Nogo-A/B (+/-) mouse. **L** depicts the lumen of the vessels and arrows mark gene expression in endothelium and smooth muscle, respectively. Whole mount staining of the heart demonstrating gene expression in large vessels, main coronary artery, left anterior descending vessel and atria (**F**). Cross section analysis demonstrates Nogo-A/B expression in atrial myocytes (**G**), coronary vessels (**H**) and vessels (black arrow) and neurons (open arrow) in the cerebral cortex (**I**).

Figure 4. The extent of vascular injury is markedly increased in Nogo-A/B knockout mice. **A.** Immunohistochemistry of Nogo-B protein shows that it is present in

all layers of non-injured vessels and is reduced in vessel one week post-injury. **B.** H&E and elastic von Giessen (EVG; to delineate the elastic laminae) staining of femoral arteries obtained from control and Nogo -A/ B (-/-) mice. **C.** Quantitative morphometry of intimal area and intima/media ratios in control and Nogo-A/B (+/- and -/-) mice at 2 weeks post-injury. Data are expressed as mean \pm SEM with 10 sections analyzed from 5 mice, * $p < 0.05$). **D.** The loss of Nogo-B promotes cellular proliferation. BrdU immunostaining in vessels from control and Nogo-A/B (-/-) mice at 2 weeks post-injury. Scale bar, 50 μ m. **E.** Quantitation of BrdU labeling in different layers (intima, media, adventitia) or whole femoral arteries at 2 weeks after injury. Data are expressed as mean \pm SEM with 10 sections of 5 arteries analyzed, * $p < 0.05$.

Figure 5. Ad Nogo-B prevents injury evoked neointimal expansion A.

Characterization of adenovirus expression HA-tagged Nogo-B. EA.hy.926 cells were infected with increasing multiplicity of infection (MOI) of a virus encoding Nogo-B. Cell extracts were blotted for Nogo-B and the HA epitope. **B.** Hematoxylin and eosin (H&E) staining of Ad β -gal and Ad-Nogo-B transduced vessels. Note the thickened neointima in Ad β -gal transduced segments compared to Ad Nogo-B transduced arteries. Scale bar represents 100 μ m **C.** Ad Nogo-B reduces injury evoked neointima. Intimal area and intima/media ratios were quantified morphometrically in vessels three weeks after injury. Data are expressed as mean \pm SEM with 10 sections analyzed from 5 mice treated with Ad β -gal or Ad Nogo-B viruses, $p < 0.05$. **D.** Ad Nogo-B reduces vascular cellular proliferation. BrdU immunostaining in Ad β -gal and Ad Nogo-B transduced vessels at 1 week after injury. **E.** Quantitation of BrdU labeling in different layers

(intima, media, adventitia) or whole femoral arteries, 1 week after injury. Data are expressed as mean \pm SEM with 10 sections analyzed from 4 mice treated with Ad β -gal or Ad Nogo-B viruses; $p < 0.05$. Scale bar represents 50 μ m.

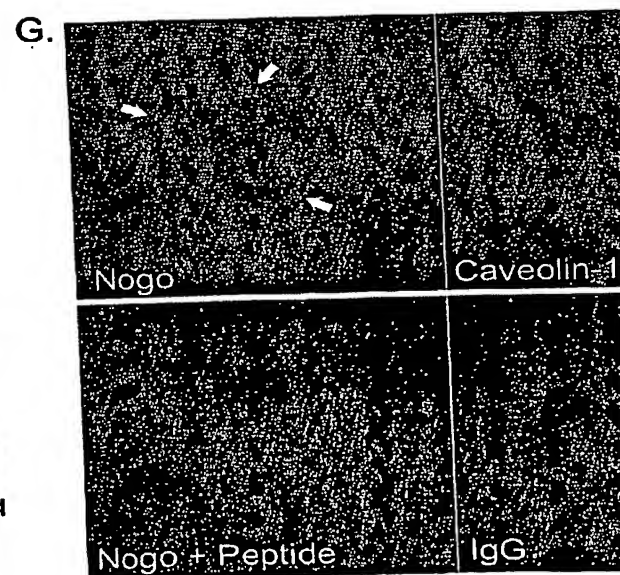
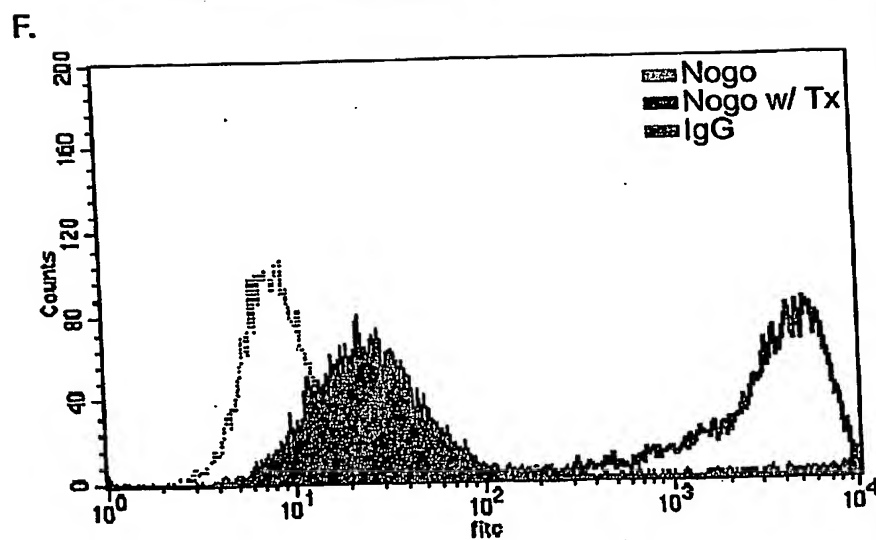
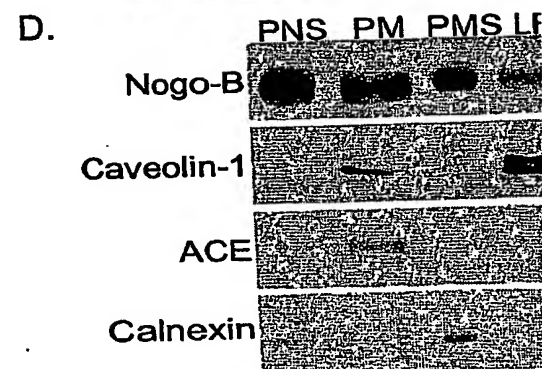
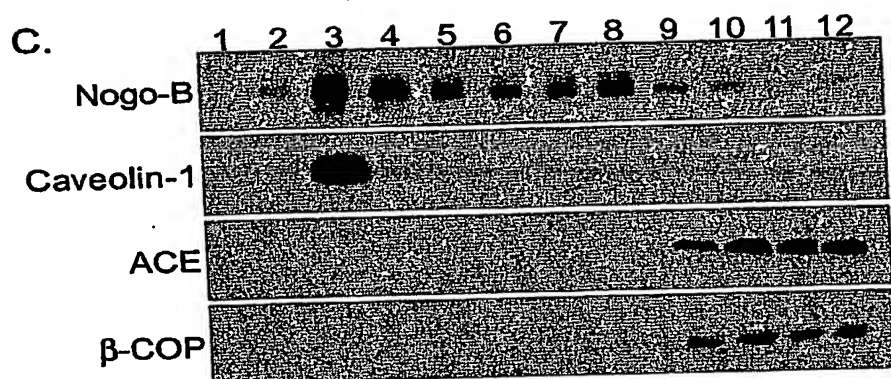
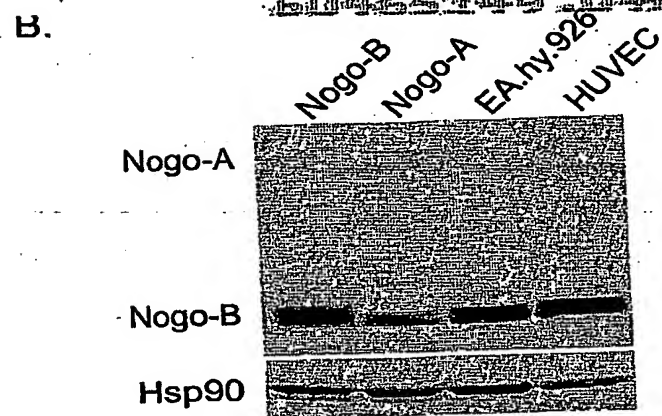
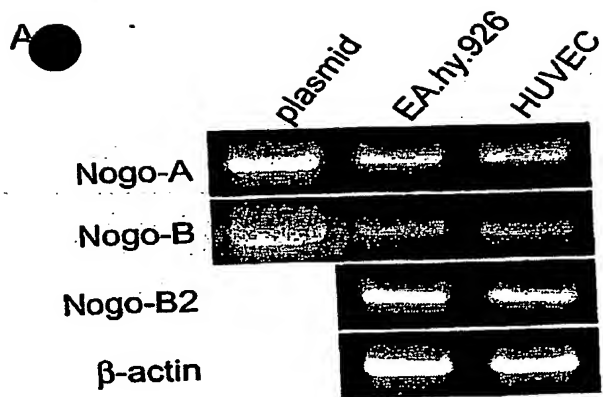
Acknowledgements

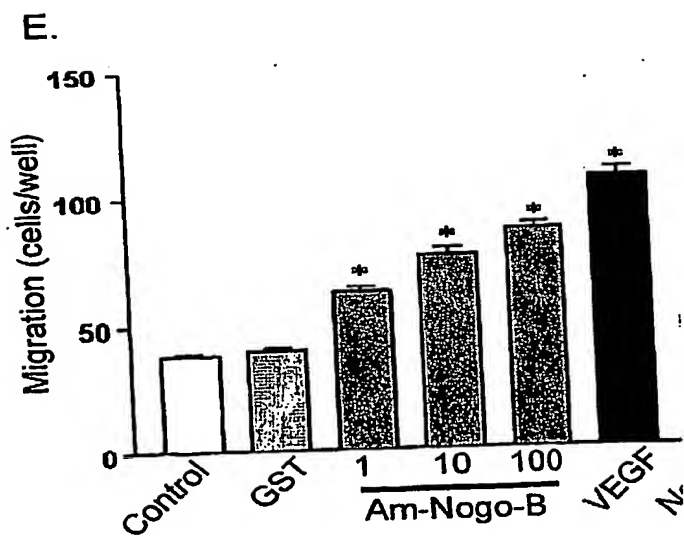
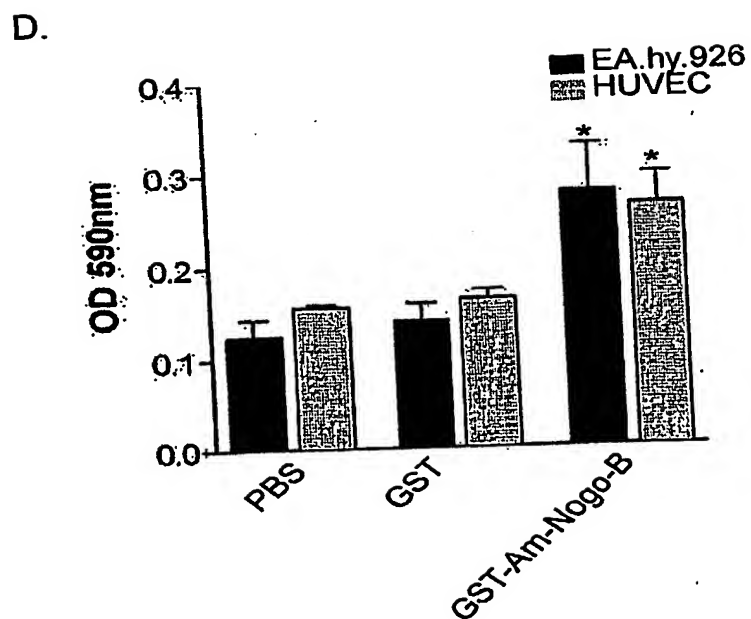
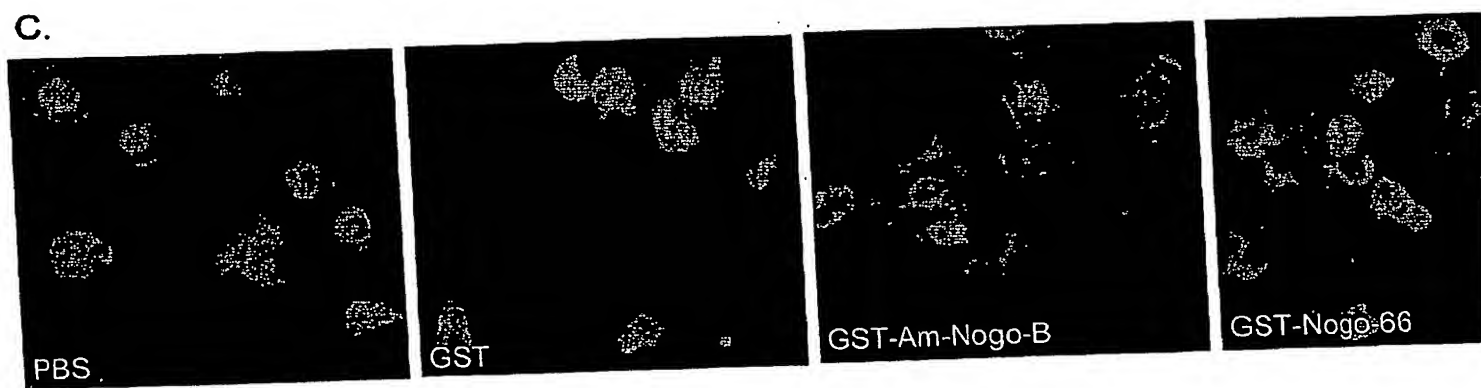
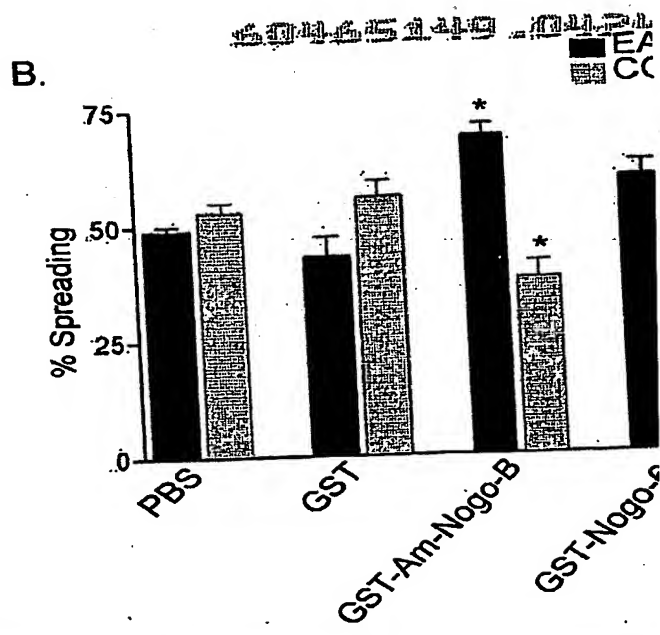
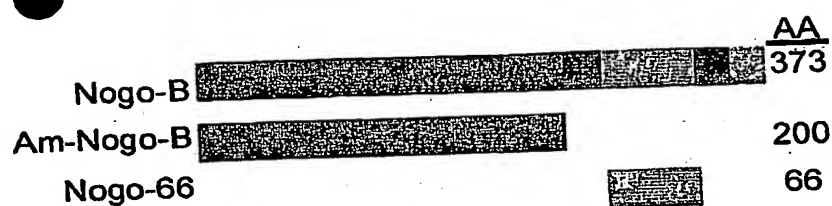
This work is supported by grants to W.C.S from the NIH.

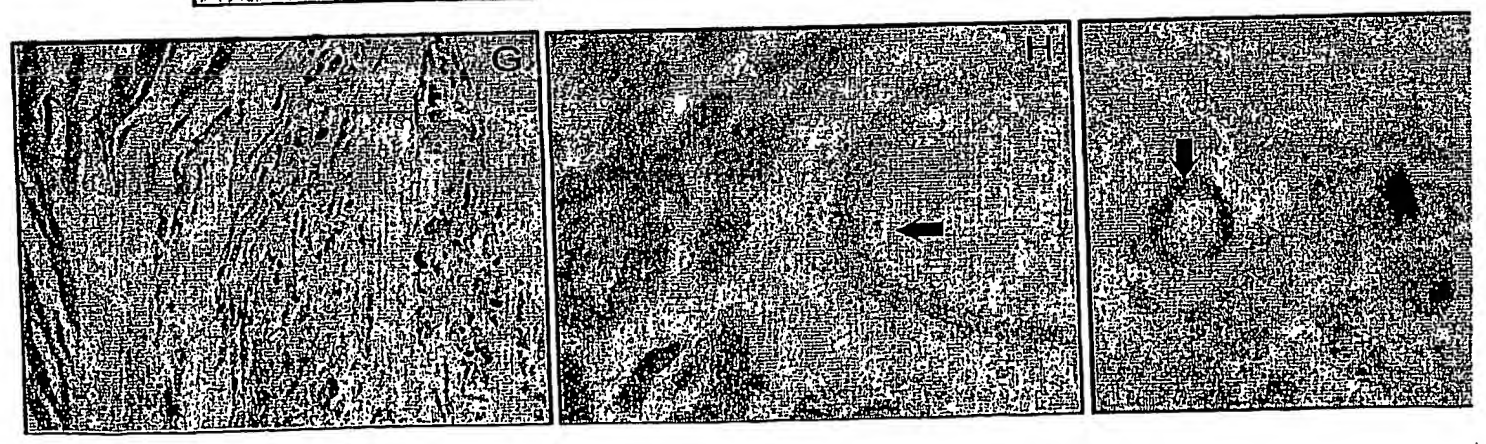
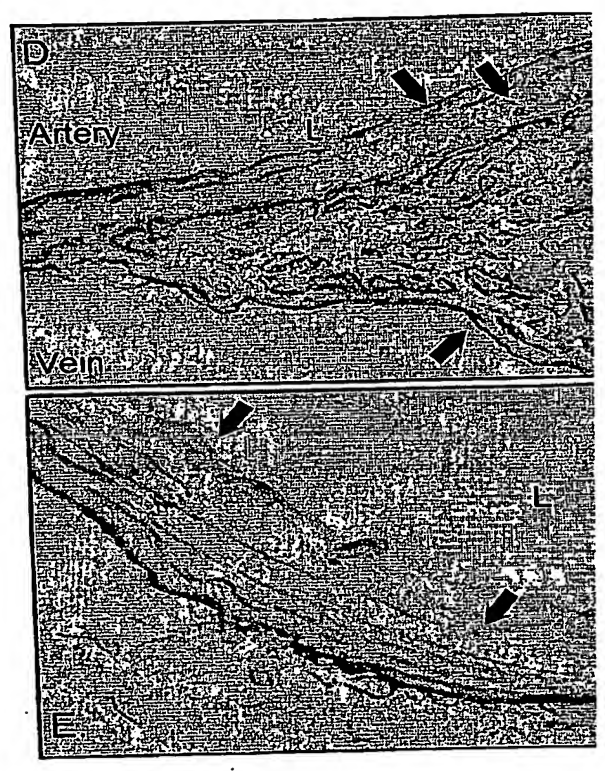
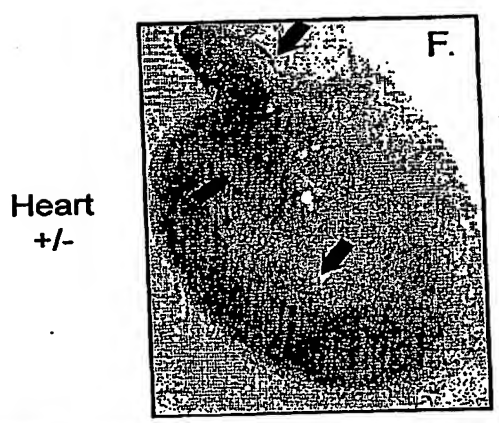
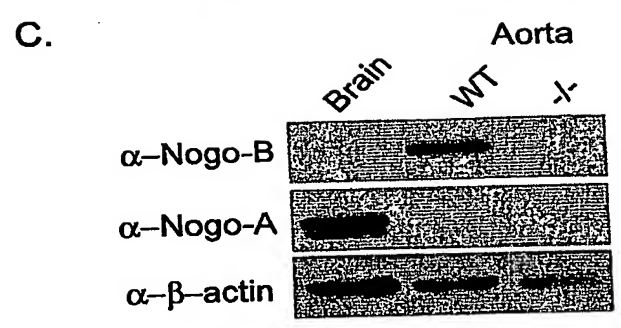
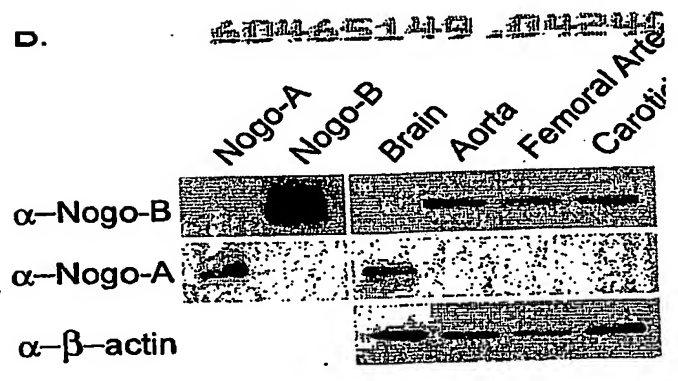
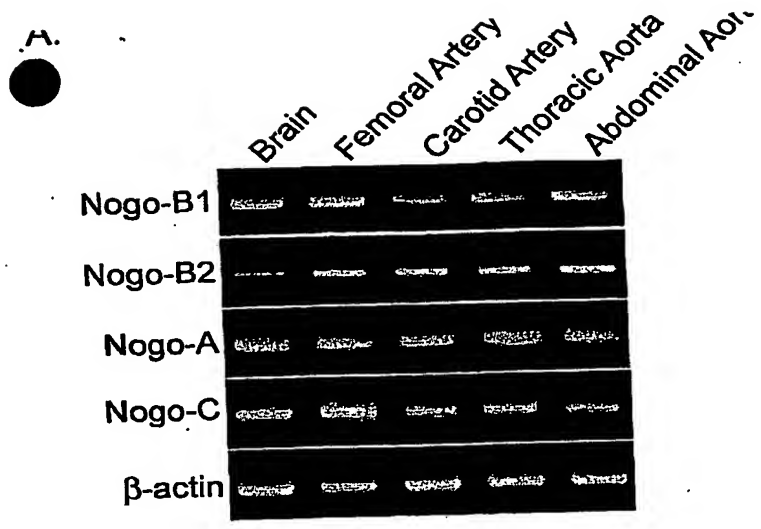
References

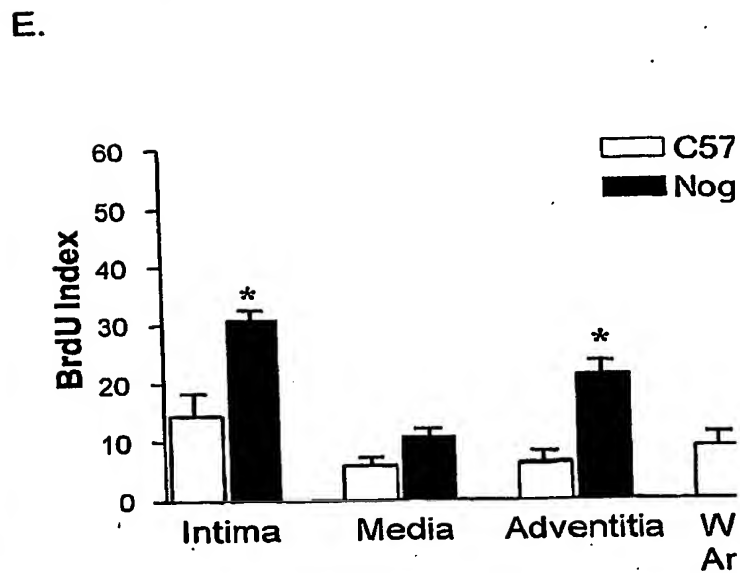
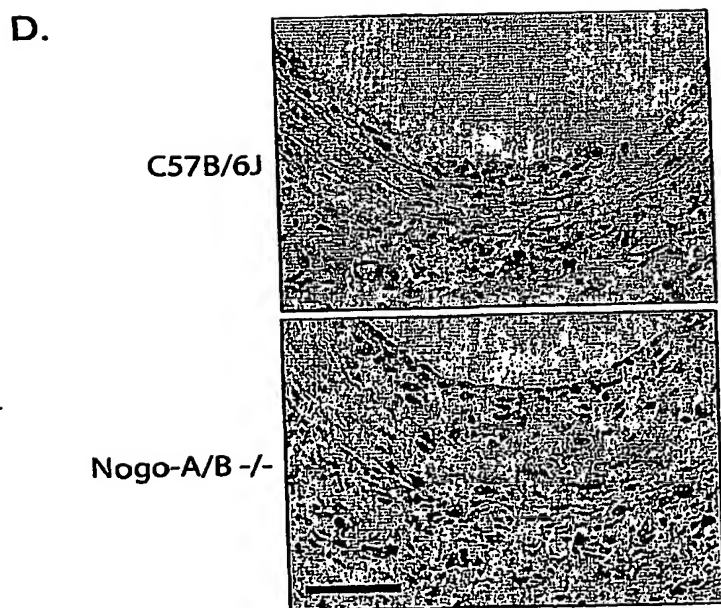
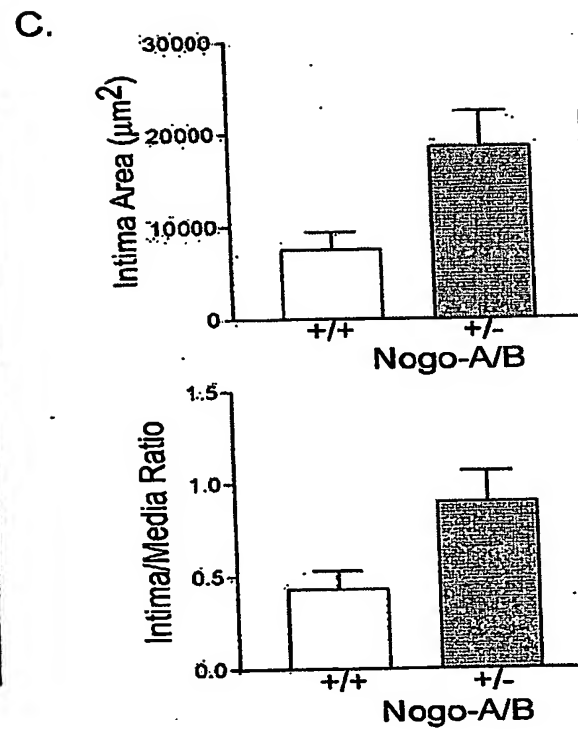
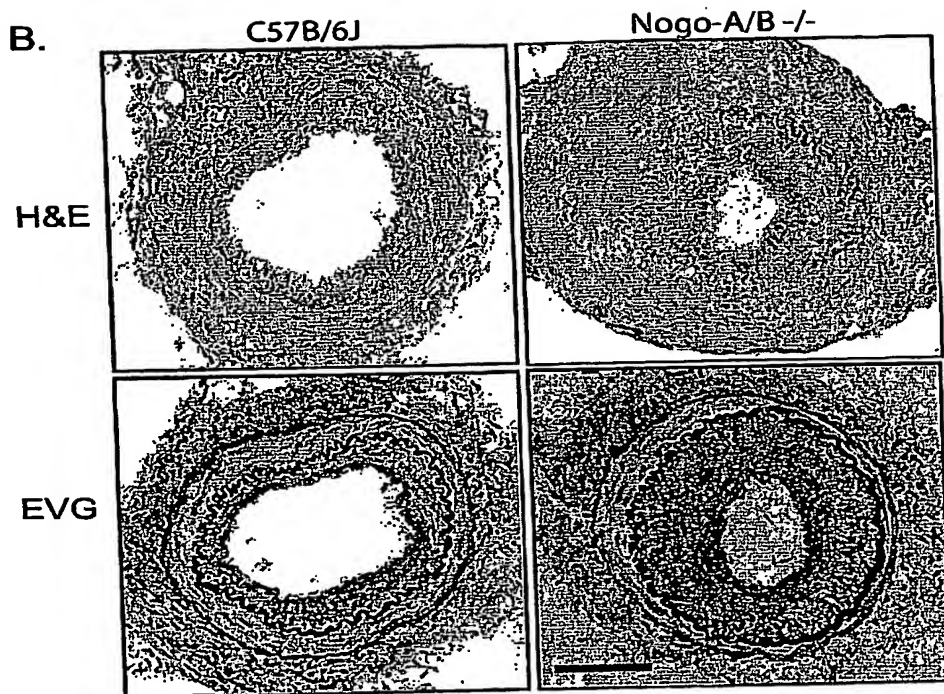
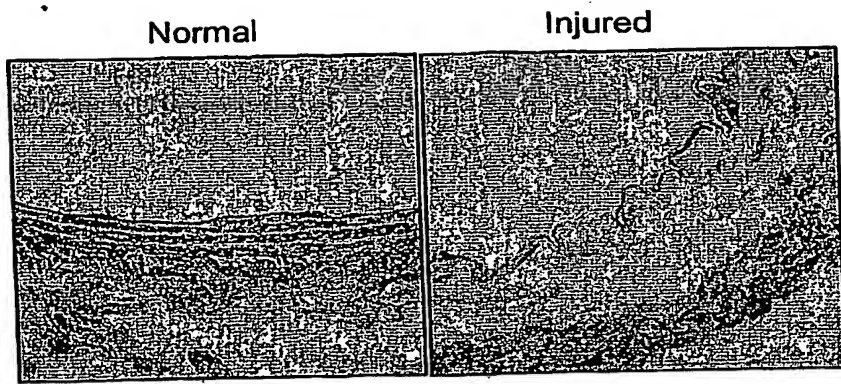
1. GrandPre, T., et al., *Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein*. Nature, 2000. 403(6768): p. 439-44.
2. Chen, M.S., et al., *Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1*. Nature, 2000. 403(6768): p. 434-9.
3. Brosamle, C., et al., *Regeneration of lesioned corticospinal tract fibers in the adult rat induced by a recombinant, humanized IN-1 antibody fragment*. J Neurosci, 2000. 20(21): p. 8061-8.
4. Merkler, D., et al., *Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A*. J Neurosci, 2001. 21(10): p. 3665-73.
5. GrandPre, T., S. Li, and S.M. Strittmatter, *Nogo-66 receptor antagonist peptide promotes axonal regeneration*. Nature, 2002. 417(6888): p. 547-51.
6. Oertle, T., et al., *Genomic structure and functional characterisation of the promoters of human and mouse nogo/rtn4*. J Mol Biol, 2003. 325(2): p. 299-323.
7. Fournier, A.E., T. GrandPre, and S.M. Strittmatter, *Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration*. Nature, 2001. 409(6818): p. 341-6.
8. Kim, J., et al., *Axon regeneration in young adult mice lacking Nogo-A/B*. Neuron.
9. He, T.-C., et al., *A simplified system for generating recombinant adenoviruses*. Proc Natl Acad Sci U S A, 1997. 95: p. 2509-2514.
10. Wang, X., et al., *Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact*. J Neurosci, 2002. 22(13): p. 5505-15.
11. Song, K.S., et al., *Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains*. J Biol Chem, 1996. 271(16): p. 9690-7.
12. Wittschieben, B.O., et al., *A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme*. Mol Cell, 1999. 4(1): p. 123-8.
13. Otero, G., et al., *Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation*. Mol Cell, 1999. 3(1): p. 109-18.
14. Morales-Ruiz, M., et al., *Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt*. Circ Res, 2000. 86(8): p. 892-6.

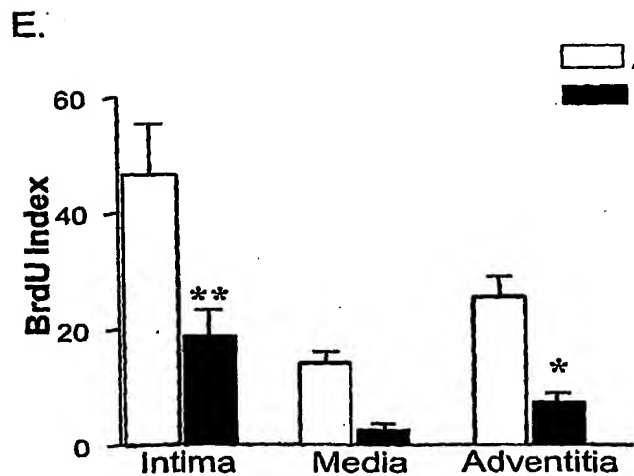
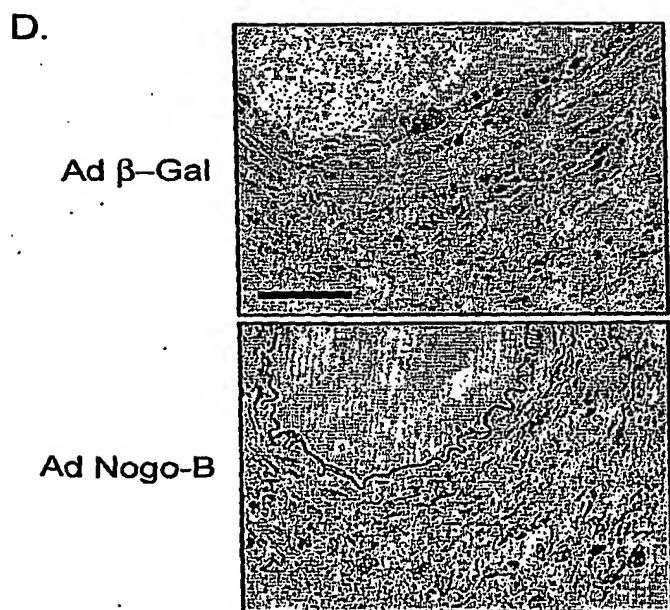
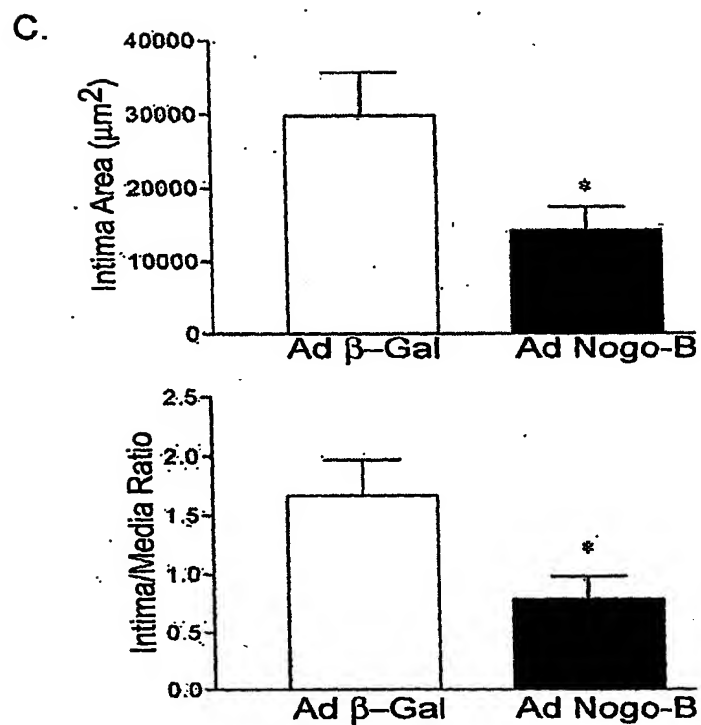
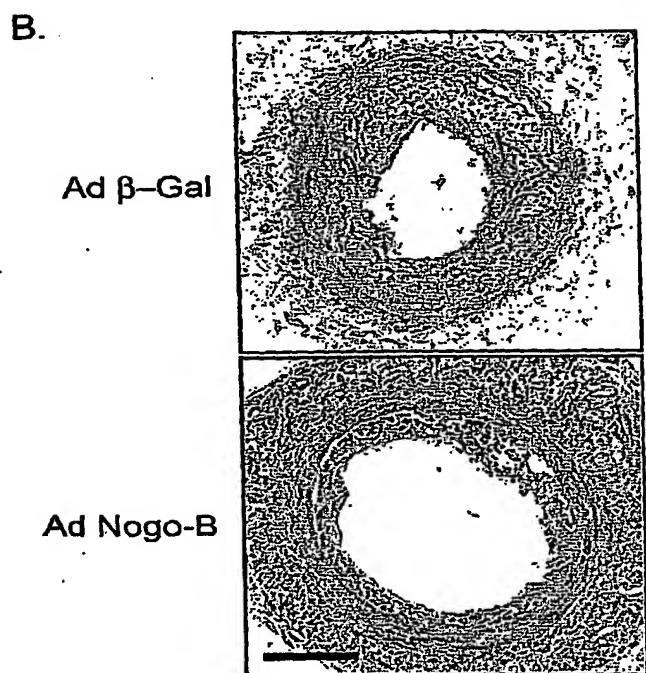
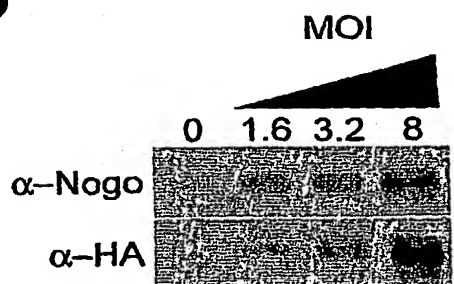
15. Ziche, M., et al., *Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis*. J Clin Invest, 1997. 99(11): p. 2625-34.
16. Blanc-Brude, O.P., et al., *Inhibitor of apoptosis protein survivin regulates vascular injury*. Nat Med, 2002. 8(9): p. 987-94.
17. Yu, J., R.D. Rudic, and W.C. Sessa, *Nitric oxide-releasing aspirin decreases vascular injury by reducing inflammation and promoting apoptosis*. Lab Invest, 2002. 82(7): p. 825-32.



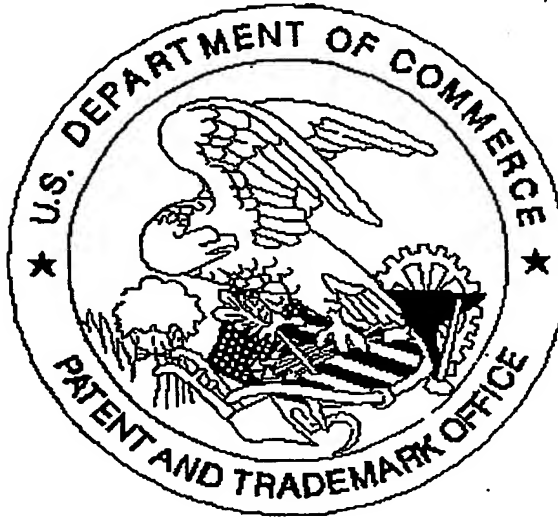








United States Patent & Trademark Office
Office of Initial Patent Examination -- Scanning Division



Application deficiencies found during scanning:

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

Only 23 pages on specification not 29

☐ **Scanned copy is best available.**

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.